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RESEARCH ARTICLE

EFFECT OF NITROGEN SOURCES, PHOSPHATE SOURCES AND METAL IONS ON THE PRODUCTION OF XYLANASE BY *BACILLUS CEREUS* BSA1

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ABSTRACT

Extracellular xylanase production by the newly isolated *Bacillus cereus* BSA1 in presence of different nitrogen and phosphate sources and metal ions was optimized under submerged fermentation. The growth of bacteria and its enzyme production were maximum in the presence of beef extract. Maximum enzyme production was done in presence of Na_2HPO_4 and the most effective concentration was achieved in 0.10%. The most effective salt for xylanase production by the bacteria was NaCl. This article says about the smart and successful production of xylanase from a mesophilic soil bacterium, *Bacillus cereus* BSA1.

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INTRODUCTION

Among the industrially used enzymes xylanase has gained paramount importance. Generally xylanases are produced by different microorganisms like fungi and bacteria through processes like solid (Amare and Gashaw, 1999; Battan *et al.*, 2006) semi solid and submerged fermentation (Gomes *et al.,* 2000). Among these processes, submerged fermentation is mostly preferred, principally because sterilization and process of control mechanisms are easier in this system (Lekha and Lonsane, 1997). Submerged fermentation involves the growth of a microorganism in a liquid medium in which nutrients are either dissolved or remain as suspended particles (Frost and Moss, 1987). Though the knowledge of microbial physiology and molecular biology reaches a desirable peak, improvement of fermentation technology remains an empirical process to obtain large quantity of desired metabolites. Nature itself provides adequate nutrients and physico-chemical environment to microorganisms for their growth and activity. Likewise an isolated organism when cultured in the laboratory should be provided with proper physico-chemical environment where it can grow in its optimum level for the maximum production of a particular metabolite of interest. A microbiologist, therefore, primarily focuses on the optimization procedure to scale up the synthesis of a desired metabolite. Nutrients as a whole serve three main functions—provide the materials required for synthesis of protoplasm, supply energy necessary for cell growth and serve as acceptors for the electrons released in the reactions that yield energy for the organism. Microbial medium basically contains sources of carbon, nitrogen, phosphate etc. and needs to be maintained in factors like required oxygen supply, acidity or alkalinity of the media, temperature etc. Microbes initially break down complex substrates into small absorbable molecules by secreting enzymes. Generally synthesis of these enzymes is induced by the complex substrate itself and the nascent enzymes are excreted out from the microbial cells. The hydrolyzed products are up taken by microbes and are exploited for the synthesis of biomolecules and energy production for maintaining the biological machineries. In the present study, chemical constituents of the submerged fermentation system like nitrogen sources, phosphate sources, metal ions for the maximum production of xylanase by previously isolated strain *Bacillus cereus* BSA1 (Mandal *et al.*, 2008)

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MATERIALS AND METHODS

Microorganism: Newly isolated bacterial strain *Bacillus cereus* BSA1 (Mandal *et al.*, 2008), was used in this study.

Production of enzyme: Enzyme productions was made in 250 ml Erlenmeyer flasks containing 50 ml of sterilized liquid media [(g/l): $(NH_4)_2SO_4$ 1.0; $MgSO_4$ 0.2; K_2HPO_4 0.2; $CaCl_2$ 0.2; $MnCl_2$ 0.02; yeast extract 0.1, xylan 10.0] Before sterilization, xylan was completely dissolved in water by sonication (7.0 hz, 2 min) and then pH of the medium was adjusted to 7.0. The medium was sterilized for 15 minutes at 121ºC. Fermentation was carried out in a rotatory shaker (120 rpm) at 35ºC for 72h. The cell-free supernatant after centrifugation (5000g× 5min) was used as the source of crude enzyme. Growth of the organism was determined according to the formation of colony forming unit (c.f.u./ml).

Optimization of fermentation process: Nutrients were optimized one after another taking one variable at a time (OVAT) for maximum xylanase production. First, different types of nutrients were considered and then their concentrations were optimized. All the experiments were done in triplicate and data were presented here as mean ± SE.

Enzyme assay: Xylanase activity was assayed by measuring released reducing sugar from birch wood xylan (Fluka) with 3, 5- dinitrosalicylic acid (Miller, 1959). The reaction mixture containing 0.4ml phosphate buffer (0.2M, pH 7.0), 0.3ml of 5% (w/v) xylan and 0.3ml enzyme solution. The enzymatic reaction was carried out at 50ºC and after 30 min 1ml of DNS (3%w/v) was added to stop the reaction. The solution was incubated in a boiling water bath for 15 min for colour development and the absorbency was measured at 540nm (Systronic spectrophotometer 105) against the enzyme blank. The xylanase activity was determined by using a standard calibration curve of D-xylose (Sigma). One unit of xylanase activity (U/ml) was defined as the amount of enzyme required to produce 1µmol of reducing sugars as xylose by hydrolyzing xylan per minute under the above assay condition.

RESULTS

Effect of different N_2 sources

Different organic and inorganic nitrogen sources (0.1% w/v) were supplemented in the culture media and their influences on enzyme production were represented in Table 1. Among the organic nitrogen sources, maximum enzyme production was noticed in presence of beef extract (6.02 ± 0.26).

Table 1. Effect of nitrogen sources on xylanase production by *Bacillus cereus* BSA1. Organism was cultivated for 84h at 35ºC and pH 6.0 under shaking (120 rev/min) condition

Nitrogen sources (0.1%, w/v)	Xylanase (U/ml)
NH_4NO_3	5.87 ± 0.40
$(NH_4)_2SO_4$	5.64 ± 0.36
NH_4Cl	4.66 ± 0.32
$NaNO_3$	3.14 ± 0.30
KNO_3	3.50 ± 0.56
$(NH_4)_2HPO_4$	4.47 ± 0.22
Peptone	5.65 ± 0.43
Yeast extract	5.32 ± 0.31
Beef extract	6.02 ± 0.26
Urea	2.33 ± 0.34
Control (Without N_2 sources)	1.04 ± 0.14

Among inorganic sources ammonium nitrate (5.87 ± 0.40) showed the best result. The increased order of enzyme production in relation to nitrogen sources can be arranged in the following order: beef extract > NH_4NO_3> peptone > $(NH_4)_2SO_4$> yeast extract > NH_4Cl > $(NH_4)_2HPO_4$ > KNO_3 > $NaNO_3$ > urea. Different concentrations of beef extract and ammonium nitrate were also tested (Table 2 and 3) separately for xylanase production and the most effective results were achieved at 0.2% (6.30 U) and 0.5% (6.10 U) respectively.

Table 2. Effect of different concentrations of beef extract on xylanase production by *Bacillus cereus* BSA1 under shaking (120 rev/min) condition for 84h. The temperature and initial medium pH were 35ºC and 6.0 respectively

% of Beef extract	Xylanase(U/ml)
0.10	6.12 ± 0.21
0.20	6.30 ± 0.18
0.30	6.01 ± 0.25
0.40	5.59 ± 0.15
0.50	5.05 ± 0.22
0.60	4.79 ± 0.16
0.70	4.41 ± 0.11
0.80	3.53 ± 0.14
0.90	3.35 ± 0.21
1.0	2.22 ± 0.16

Table 3. Study of the Effect of different concentrations of ammonium nitrate on xylanase production by *Bacillus cereus* BSA1 at 35ºC and pH 6.0 under shaking (120 rev/min) condition

% NH_4NO_3	Xylanase(U/ml)
0.10	5.81 ± 0.23
0.20	5.93 ± 0.31
0.30	6.00 ± 0.35
0.40	6.03 ± 0.12
0.50	6.10 ± 0.22
0.60	6.02 ± 0.17
0.7	5.83 ± 0.13
0.8	4.63 ± 0.11
0.9	4.10 ± 0.21
1.00	3.21 ± 0.16

Effect of Phosphate Sources

Effects of inorganic phosphates (0.02% w/v) on the production of xylanase by *B. cereus* BSA1 were studied and represented in Table 4. Among the tested phosphate sources maximum xylanase production (5.53 ± 0.47 U/ml) occurred in presence of Na_2HPO_4 and the most effective concentration was achieved in 0.10% (Table 5).

Table 4. Effect of different phosphate sources on xylanase production by *Bacillus cereus* BSA1. Fermentation was carried out for 84h at 35ºC and pH 6.0 under shaking (120 rev/min) condition

Phosphate source (0.02%)	Xylanase(U/ml)
K_2HPO_4	5.38 ±0.21
KH_2PO_4	4.80 ± 0.33
NaH_2PO_4	4.84 ± 0.30
$NH_4H_2PO_4$	1.07 ±. 41
$(NH4)_2HPO_4$	4.07 ± 0.29
Na_2HPO_4	5.53 ± 0.47
Control (Without phosphate sources)	1.56 ± 0.33

Table 5. Study of effective concentration of Na_2HPO_4 on xylanase production by *Bacillus cereus* BSA1 at 35ºC and pH 6.0 under shaking (120 rev/min) condition for 84h

% of Na_2HPO_4	Xylanase(U/ml)
0.025	5.51 ± 0.22
0.050	5.87 ± 0.11
0.075	6.21 ± 0.25
0.100	6.33 ± 0.17
0.125	5.80 ± 0.33
0.25	3.42 ± 0.12
0.50	2.71 ± 0.15

Effect of metal ions on xylanase production

Effect of different metal ions like manganese, mercury, calcium, sodium, potassium, magnesium, ferric (0.5% w/v) were tested (in the form of chloride salt) for the production of the enzyme. Maximum production of enzyme was recorded in presence of 0.5% sodium chloride (Table 6) Enzyme production was found to be inhibited by mercury and ferric ions. The order of enzyme production in relation to the metal ions can be arranged in the following order: NaCl> $MnCl_2$ > $CaCl_2$ >KCl> $MgCl_2$ >$FeCl_3$ > $HgCl_2$ (Table 6). The most effective concentration of NaCl for xzylansaebiosaynthesis was achieved in 0.50% (Table 7).

Table 6. Effect of metal ions on xylanase production by *Bacillus cereus* BSA1 Different salt were mixed separately with the media and the organism was grown at 35ºC and pH 6.0 under shaking (120 rev/min) condition for 84h

Metal ion sources (0.5%)	Relative activity of xylanase (%)
NaCl	100
$HgCl_2$	0.08
$MnCl_2$	97
$CaCl_2$	93
KCl	92
$MgCl_2$	85
$FeCl_3$	69
Control	84

Table 7. Study of xylanase production by *Bacillus cereus* BSA1 in different concentrations of NaCl. The organism was grown at 35ºC and pH 6.0 under shaking (120 Rev/Min) condition for 84h

% of NaCl	Xylanase(U/ml)
0.05	6.01 ± 0.23
0.10	6.13 ± 0.32
0.20	6.34 ± 0.11
0.30	6.48 ± 0.23
0.40	6.57 ± 0.25
0.5	6.71 ± 0.22
0.6	6.44 ± 0.23
0.7	6.35 ± 0.11
0.8	5.92 ± 0.23
1.0	5.57 ± 0.17

DISCUSSION

In the study, beef extract, a balanced source of protein in terms of composition and accessibility, showed higher stimulatory effect for xylanase biosynthesis. This complex natural product contained different kind of peptides and other useful ingredients, which collectively favoured growth of bacteria as well as its enzyme production. Kohli *et al*. (2001) obtained higher amount of xylanase production by *Thermoactinomycesthalophilus* in presence of complex organic nitrogen sources like yeast extract. Among the inorganic nitrogen sources ammonium nitrate had comparatively higher stimulating effect than other. The phosphates are very important nutrient for bacterial growth. Effects of different inorganic phosphates have been studied for enzyme production and it was found that 0.1% (w/v) of Na_2HPO_4 was the best inducer for bacterial growth and enzyme production. Phosphates are generally required in the microbial cell as an energy source, but Priest (1977) assumed that phosphates might increase mRNA stability by inhibiting RNAase activity and thereby increase the enzyme production. Again increased amount of phosphate source caused decreased xylanase production. This indicated that, available phosphate source induced growth of the organism by enhancing the cellular energy pool, which repress the enzyme biosynthesis like other operon systems.

The bacterium grew in presence of different types of metal ions. Actually, micro and macro elements are required for elementary composition of all living cells, but particular ion has got stimulatory effect to metabolic pathway in a specific group of microorganisms. The presence of NaCl in culture media mostly stimulated enzyme production and this might develop a particular membrane potential that favoured enzyme release from the cell. Studies have shown interest on the unique structural and biochemical characteristics of exoenzyme regarding their relation with salt (Ryu *et al*., 1994; Kim and Dordick, 1997; Ru *et al*., 1999, Lee *et al*., 2006) and their potentialities in many industrial applications (Flam, 1994; Adams *et al*., 1995; Ventosa and Nieto, 1995). Decrease in xylanase production in presence of Hg^{++} ion may be nonspecific binding or aggregation of this ion with some essential enzymes. They may also cause a reduction in catalytic activity due to partial denaturation of enzyme (Tunga *et al*., 1999).

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